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Research

Chemical priming of immunity without costs to plant growth

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Summary

- β-Aminobutyric acid (BABA) induces broad-spectrum disease resistance, but also represses plant growth, which has limited its exploitation in crop protection. BABA perception relies on binding to the aspartyl-tRNA synthetase (AspRS) IBI1, which primes the enzyme for secondary defense activity. This study aimed to identify structural BABA analogues that induce resistance without stunting plant growth.
- Using site-directed mutagenesis, we demonstrate that the (L)-aspartic acid-binding domain of IBI1 is critical for BABA perception. Based on interaction models of this domain, we screened a small library of structural BABA analogues for growth repression and induced resistance against biotrophic *Hyaloperonospora arabidopsidis* (*Hpa*).
- A range of resistance-inducing compounds were identified, of which (*R*)-β-homoserine (RBH) was the most effective. Surprisingly, RBH acted through different pathways than BABA. RBH-induced resistance (RBH-IR) against *Hpa* functioned independently of salicylic acid, partially relied on camalexin, and was associated with augmented cell wall defense. RBH-IR against necrotrophic *Plectosphaerella cucumerina* acted via priming of ethylene and jasmonic acid defenses. RBH-IR was also effective in tomato against *Botrytis cinerea.* Metabolic profiling revealed that RBH, unlike BABA, does not majorly affect plant metabolism.
- RBH primes distinct defense pathways against biotrophic and necrotrophic pathogens without stunting plant growth, signifying strong potential for exploitation in crop protection.

Introduction

Plants rely on their innate immune system to resist microbial pathogens. This defense regulatory system controls a wide range of pathogen-inducible defense mechanisms, such as transcriptional activation of defense genes, production of secondary metabolites and structural reinforcements of the cell wall (Jones & Dangl, 2006). A range of small signaling molecules, including reactive oxygen species (ROS), salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), play critical roles in the coordination of these inducible defenses (Pieterse *et al.*, 2012). In addition to innate immunity, plants also can acquire immunity upon perception of specific biotic and abiotic stimuli, a process mediated largely by priming of inducible defenses (Conrath *et al.*, 2006). Immune priming enables faster and/or stronger induction of inducible defenses following subsequent pathogen attack. A classic example is systemic acquired resistance (SAR), whereby localized pathogen attack primes SA-dependent defenses in distal tissues (Jung *et al.*, 2009). Interactions with beneficial soil microbes, such as growth-promoting rhizobacteria and fungi, can also elicit systemic priming of JA- and ET-dependent defenses (Verhagen *et al.*, 2004; Van der Ent *et al.*, 2009), which is commonly referred to as induced systemic resistance (ISR; Van Wees *et al.*, 2008) or mycorrhiza-induced resistance (MIR; Cameron *et al.*, 2013). Because priming augments multigenic basal resistance, the resulting disease protection can be more durable than race-specific resistance, which is based on single resistance genes (Ahmad *et al.*, 2010). Therefore, despite the fact that priming rarely provides complete disease protection (Walters *et al.*, 2013), application of priming-inducing agents is increasingly considered for exploitation in integrated pest and disease management (Beckers & Conrath, 2007; Conrath *et al.*, 2015).

β-amino butyric acid (BABA) is a well-known chemical priming agent that induces broad-spectrum disease resistance in a wide range of economically important crop species (Cohen *et al.*, 2016). BABA-induced resistance (BABA-IR) is based on priming of multiple defense mechanisms that are controlled by SA-dependent and SA-independent pathways (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). Recently, BABA was found to occur naturally at low concentrations in plant tissues (1–20 ng g^{-1} FW), which can increase five- to 10-fold upon exposure to (a) biotic stress (Thevenet *et al.*, 2016). Consistent with a function as an endogenous defense regulatory signal, we previously discovered a receptor protein for BABA (Luna *et al.*, 2014). A genetic screen for

Arabidopsis thaliana (Arabidopsis) mutants in BABA-IR identified the *IMPAIRED IN BABA-INDUCED IMMUNITY 1* (*IBI1*) gene, which encodes an aspartyl-tRNA synthetase (AspRS). Binding of the active (*R*)-enantiomer of BABA to IBI1 primes this enzyme for alternative defense activity in the cytoplasm. Despite the documented ability of BABA to protect against a wide range of commercially relevant crop diseases, agricultural exploitation of the chemical has been hampered by its growth-repressing effects (van Hulten *et al.*, 2006; Wu *et al.*, 2010). This phytotoxicity is caused by the inhibitory activity of BABA on AspRS enzyme activity (Luna *et al.*, 2014), which is aggravated by its slow metabolic turnover in the plant (Jakab *et al.*, 2001; Slaughter *et al.*, 2012). Consequently, treatment of plants with BABA progressively blocks AspRS activity, causing cellular accumulation of uncharged $tRNA^{Asp}$, GCN2-dependent repression of gene translation, and plant stress (Luna *et al.*, 2014). Because the chemical structures of (L)-Asp and (*R*)-BABA are strikingly similar, we proposed that this AspRS-inhibiting effect of (*R*)-BABA is due to aspecific binding to the (L)-Asp binding site of AspRS proteins. This mode of action explains why mutants in *IBI1* are not only impaired in BABA-IR, but also hypersensitive to BABA-induced stress. The reduced concentrations of AspRS protein in *ibi1* plants lowers BABA sequestration capacity, causing increased accumulation of un-charged $tRNA^{Asp}$, and enhanced stress after BABA treatment (Luna *et al.*, 2014).

In the present study, we took a chemical approach to identify resistance-inducing analogues of BABA with fewer nontarget effects on plant growth. Based on ligand-interaction models of the conserved (L)-Asp-binding domain of IBI1, we screened a small library of β-amino acids for resistance-inducing activity and growth repression. We identified seven resistance-inducing compounds, of which five acted independently of AspRS inhibition. *(R)*-β-homoserine (RBH) showed the strongest resistance-inducing activity, which acted via partially different signaling pathways than BABA, without affecting vegetative growth or global plant metabolism. Because RBH protects taxonomically unrelated plant species against biotrophic and necrotrophic pathogens, we conclude that RBH represents a promising new agent in crop protection strategies.

Materials and Methods

Plant material and growth conditions

Details of *Arabidopsis thaliana* (L.) Heynh. genotypes are provided in the Supporting Information Methods S1. Surface-sterilized seeds were sown on Murashige & Skoog agar (1.5%), or in pots containing a 2 : 1 (v/v) peat : sand mixture. Seeds were stratified at 4°C in darkness for 2 d before cultivation under short-day growth conditions (8 h (150 μmol photons m^{-2} s^{-1}) 21°C : 16 h 18°C, day : night) at *c.* 60% relative humidity (RH). Tomato seeds (*Solanum lycopersicum* (L.) cultivar Micro-Tom) were sown in petri dishes on wetted filter paper and kept at 28°C until germinated. Once germinated, seedlings were transplanted into pots with Scott's Levington M3 soil and were cultivated under long-day conditions (16 h (150 μmol photons m^{-2} s^{-1}) 23°C : 8 h 20°C, day : night) at *c.* 60% RH.

Chemical treatments

Details of the 10 chemicals of our chemical library are provided in Methods S2. Soil-drench treatment with chemicals was performed by injecting a 10× concentrated solution at 10% of the pot volume. Lids were removed for 1 d after treatment to increase solution uptake by soil. Chemical concentrations in agar media were as indicated in figures.

Site-directed mutagenesis and interaction modeling

A point mutation converting the glutamine residue (GLN308) of the (L)-Asp-binding site in IBI1 to a nonpolar alanine residue (ALA308) was introduced by site-directed mutagenesis, as described in Methods S3. Modelling of ligand interactions between β-amino acids and the (L)-Asp binding domain of IBI1 was based on *Thermococcus kodakaraensis* AspRS co-crystalized with (L)-Asp (PDB:3NEL), as explained previously (Luna *et al.*, 2014). Simulations were performed using the Discovery Studio (DS) (Accelrys Software Inc., San Diego, CA, USA) platform, as detailed in the Methods S4.

Pathogen strains and induced resistance assays

Induced resistance in Arabidopsis was quantified against the biotrophic oomycete *Hyaloperonospora arabidopsidis*, strain WACO9 (Col-0 assays) or CALA (L*er* assays), and the necrotrophic fungus *Plectosphaerella cucumerina* strain BMM. Induced resistance in tomato was quantified against the necrotrophic fungus *Botrytis cinerea* strain R16. Details of induced resistance assays are presented in Methods S5.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

Snap frozen leaf tissues were homogenized in 2-ml tubes containing three metal beads. RNA isolation, reverse transcription and qPCR were performed as described previously (López Sánchez *et al.*, 2016). Relative transcript quantities were calculated according to $(1 + E)^{\Delta Ct}$, where $\Delta Ct = Ct(sample) - Ct(calibrator\ sample)$, and normalized to $(1 + E)^{\Delta Ct}$ values of three reference genes, *At1g13440* (GAPDH), *At5g25760* (UBC) and *At2g28390* (Czechowski *et al.*, 2005).

Staining for callose deposition against *Hpa*

Deposition of *Hpa*-induced callose was examined at 2 d post-inoculation (dpi) in aniline blue/calcofluor-stained leaves, using UV epi-fluorescence microscopy as described previously (Ton *et al.*, 2005). Effectiveness of callose deposition was quantified by the percentage of germinated conidiospores of which the proximal end of the emerging germ tube was encapsulated in callose.

Quantification of basal callose deposition in unchallenged leaves was performed as described previously (Luna *et al.*, 2011).

Stress and relative growth rate (RGR) assays

Phenotypic stress symptoms by β-amino-acids were recorded by digital photography of seedlings at 11 d after planting surface-sterilized seeds on control- (water) or β-amino-acid-supplemented 1.5% MS agar (10 g sucrose, 5 g MES, 4 g MS, 15 g Bacto-agar l^{-1}, pH 5.7). Relative growth rate (RGR) was determined as described in Methods S6.

Liquid chromatography coupled to mass spectrometry analysis

Profiling of defense-related metabolites in mock- and *Hpa*-inoculated plants was performed by ultra-performance liquid chromatography coupled to triple quadrupole mass spectrometry (UPLC-TQD; Table S1), as described previously (Gamir *et al.*, 2012). Details of untargeted metabolite profiling of water-, BABA- and RBH-treated plants by ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry (UPLC-Q-TOF), and quantification of L-Aspartic acid-derived amino acids in water- and RBH-treated plants by hydrophilic interaction liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry (HILIC-Q-TOF) are described in Methods S7 and S8.

Results

The L-Asp-binding domain of IBI1 is essential for plant perception of BABA

Based on previous models of the interaction between IBI1 and (*R*)-BABA, we predicted that the polar glutamine residue in the L-Asp-binding domain interacts with both the carboxyl and amino group of (*R*)-BABA (Luna *et al.*, 2014). To confirm the importance of the L-Asp-binding domain in BABA perception, we used site-directed mutagenesis to replace the glutamine residue in this domain with a nonpolar alanine. After transformation of *ibi1-1* with constitutively expressed (WT) (*35S:IBI1-YFP*) or mutant (*35S:IBI1$_m$-YFP*) constructs, T2 plants were compared to WT (Col-0) and *ibi1-1* plants for BABA tolerance and BABA-IR against biotrophic *H. arabidopsidis* (*Hpa*). YFP-fluorescent T_2 plants expressing WT *IBI1-YFP* fully complemented the *ibi1-1* mutant for tolerance on BABA-containing agar (0.5 mM; Figs 1a, S1). By contrast, fluorescent T_2 plants expressing mutant *IBI1$_m$-YFP* showed a comparable delay in seed germination and stunted growth as *ibi1-1* plants (Figs 1a, S1). Furthermore, fluorescent T_2 plants expressing WT IBI1-YFP complemented the *ibi1-1* mutant for BABA-IR, whereas fluorescent T_2 plants expressing mutated IBI1$_m$-YFP failed to develop statistically significant levels of BABA-IR (Fig. 1b). Hence, the L-Asp-binding domain of IBI1 is essential for BABA perception.

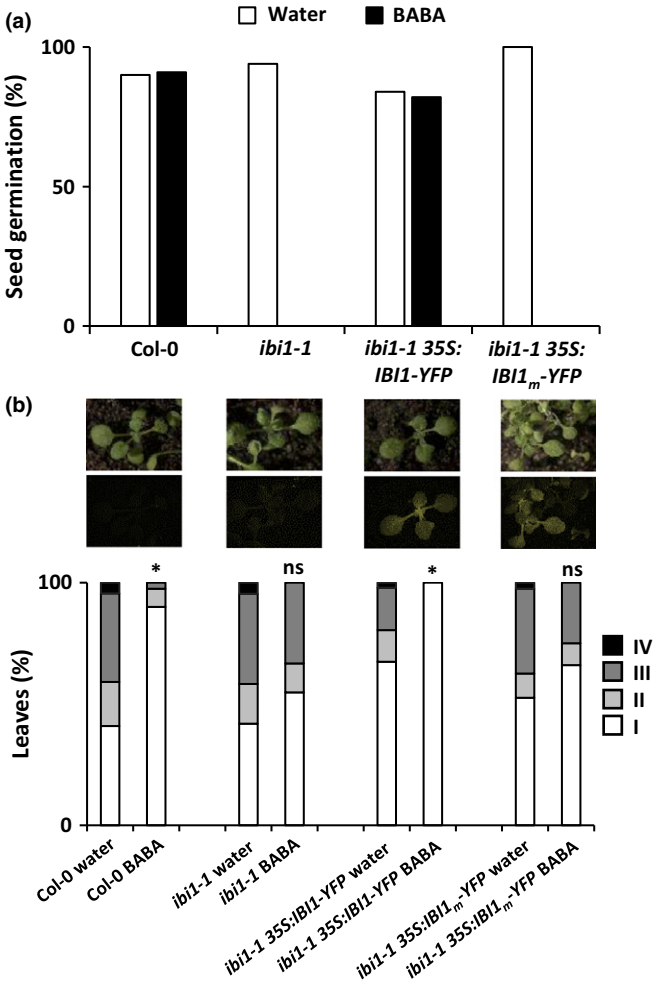

Fig. 1 The L-Asp-binding domain of IMPAIRED IN BABA-INDUCED IMMUNITY 1 (IBI1) is essential for β-aminobutyric acid (BABA) tolerance and BABA-induced resistance against *Hyaloperonospora arabidopsidis* (*Hpa*) in *Arabidopsis thaliana*. The *ibi1-1* mutant was transformed with *p35S:IBI1-YFP* constructs with and without a point mutation that converts the polar glutamine residue in the L-Asp-binding domain into a nonpolar alanine. To ensure sufficient expression of transgenes, only yellow fluorescent protein (YFP)-fluorescent T_2 individuals were selected for analysis. (a) Relative germination rates of Col-0, *ibi1-1*, *ibi1-1 35S:IBI1-YFP* and *ibi1-1 35S:IBI1$_m$-YFP* carrying the point mutation. Shown are percentages of germinated seedlings ($n = 15$–25) on MS agar with and without BABA (0.5 mM) at 6 d after planting. (b) BABA-IR against *Hpa* in Col-0, *ibi1-1*, *ibi1-1 35S:IBI1-YFP* and *ibi1-1 35S:IBI1$_m$-YFP* plants. Two-week-old plants were soil-drenched with water or BABA (0.1 mM) and challenge-inoculated with *Hpa*. Colonization by *Hpa* was analyzed microscopically at 6 d post-inoculation (dpi) in trypan blue-stained leaves by assigning leaves to different classes (see Fig. 2 for representative examples), ranging from I (no hyphal colonization) to IV (extensive hyphal colonization and formation of (a)sexual spores). Statistically significant differences in class distributions (Fisher's exact test) compared to the water-treated controls are indicated: *, $P < 0.05$; ns, not significant. Photos of representative plants were taken at 23 d after planting in bright light (upper panels) and YFP-fluorescent light (lower panels).

In silico interaction models between β-amino acids and the L-Asp-binding domain of AspRS

In order to search for analogs of *(R)*-BABA, we modeled the interaction between structurally related β-amino acids and the

(L)-Asp-binding domain of IBI1. Because this domain is highly conserved between eukaryotic and prokaryotic AspRS enzymes, binding simulations were based on the co-crystallized structure of (L)-Asp-bound AspRS from *T. kodakaerensis*, as described previously (Luna *et al.*, 2014). *(S)*-β-homoserine, (L)-threo-3-methylaspartic acid, β-alanine, and the *(R)*-enantiomers of β-amino-pentanoic acid, β-amino-hexanoic acid and β-amino-heptanoic acid all docked in a similar orientation to (L)-Asp and (*R*)-BABA, which is determined by interactions of the positively charged amine group of these molecules with residue GLN308 and residues ASP348 and SER307 via a bridging water molecule (Fig. S2). By contrast, *(R)*-β-homoserine and β-glutamic acid docked in a different orientation to (*R*)-BABA and (L)-Asp, due to different positioning of their amine groups (Fig. S2).

AspRS inhibitory activity by structural BABA analogues

Arabidopsis mutants in *IBI1* are hypersensitive to BABA-induced inhibition of AspRS function (Luna *et al.*, 2014), causing delayed germination and dramatically repressed growth on BABA-containing agar (Figs 1a, S1). We exploited this mutant phenotype to screen the β-amino acids for AspRS inhibitory activity. Growth phenotypes of 1-wk-old *ibi1-1* and Col-0 seedlings were compared on agar plates containing 0.5 mM of each of the β-amino acids (Fig. 2). The analysis for threo-3-methylaspartic acid, β-amino-pentanoic acid, β-amino-hexanoic acid and β-amino-heptanoic acid was based on racemic mixtures, because no enantiomer-pure preparations of these chemicals were available. Germination and growth of Col-0 was unaffected by any of the β-amino acids (Fig. 2). As expected, BABA selectively repressed germination and growth of *ibi1-1* (Fig. 2). To a lesser extent, *ibi1-1* seedlings also showed stunted growth on plates containing *(S)*-β-homoserine and β-alanine, indicating that these compounds exert weak AspRS inhibitory activity. None of the β-amino acids repressed growth in *ibi1-1*, suggesting that they do not affect AspRS function (Fig. 2).

Induced resistance by structural BABA analogs

In order to assess the resistance-inducing activities of the β-amino acids, 2-wk-old Col-0 plants were soil-drenched with increasing concentrations of each chemical and challenged with *Hpa*. Resistance was determined 5–6 dpi by scoring *Hpa* colonization in trypan blue-stained leaves. *(S)*-β-homoserine and β-alanine induced partial resistance against *Hpa*, which was proportional to their

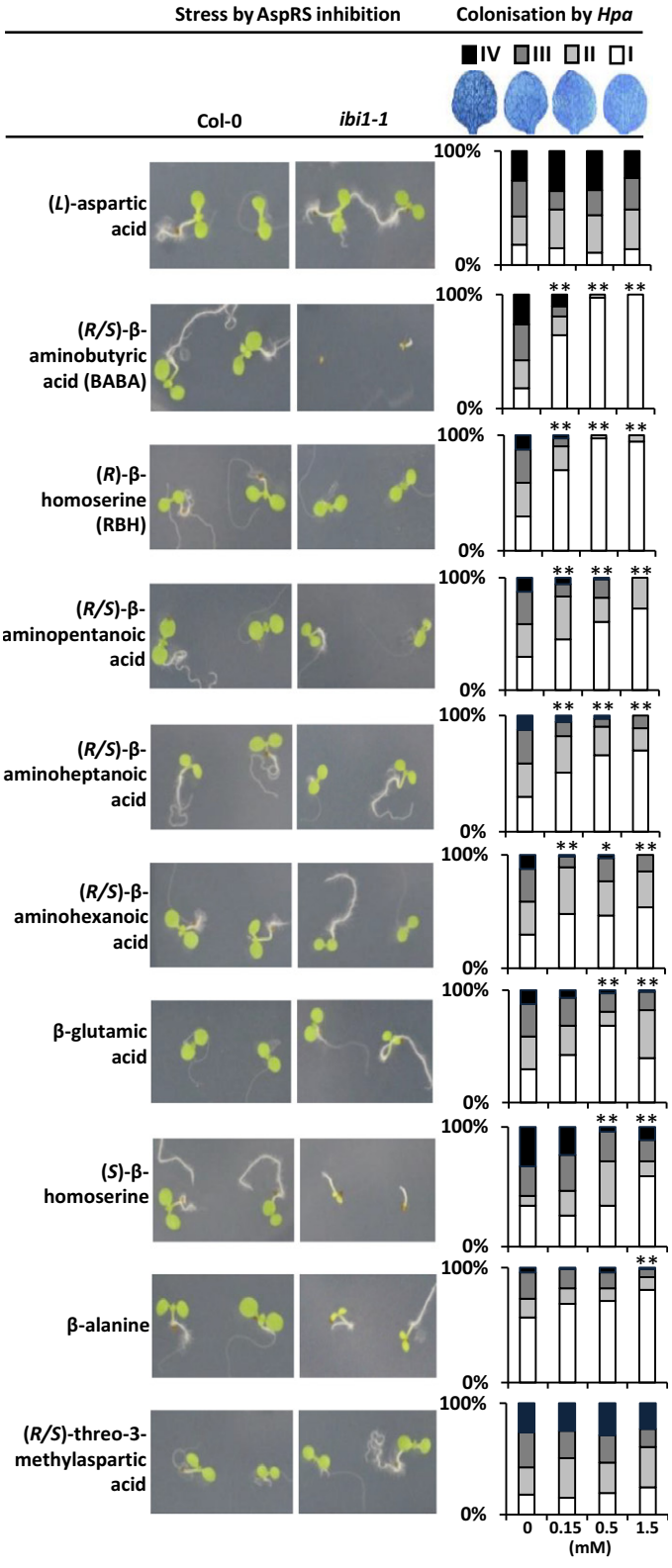

Fig. 2 Aspartyl-tRNA synthetase (AspRS) inhibitory activity (left) and resistance-inducing activity (right) of eight structural analogues of β-aminobutyric acid (BABA) in *Arabidopsis thaliana*. AspRS inhibitory activity was deduced from selective growth inhibition of *IMPAIRED IN BABA-INDUCED IMMUNITY 1* (*ibi1-1*) mutant seedlings, which signifies reduced AspRS enzyme activity (Luna *et al.*, 2014). Shown are 11-d-old wild-type (Col-0) and *ibi1-1* seedlings on Murashige & Skoog agar plates, containing 0.5 mM of each β-amino acid. Resistance-inducing activity was determined by soil-drenching 2-wk-old Col-0 with increasing concentrations of the chemicals, followed by challenge inoculation with *Hyaloperonospora arabidopsidis* (*Hpa*) 2 d later. Shown are percentages of leaves in different *Hpa* colonization classes at 5–6 d post-inoculation (dpi) ($n = 70–80$). Insets show representative examples of *Hpa* colonization classes. Statistically significant differences in class distributions (Fisher's exact test) compared to the water-treated controls (0 mM) are indicated: *, $0.01 < P < 0.05$; **, $P < 0.01$).

growth-repressing effects on *ibi1-1* (Fig. 2), indicating that these molecules act as weak functional analogs of BABA. *(D/L)*-threo-3-methylaspartic acid failed to induce resistance against *Hpa*, consistent with its inability to induce selective growth inhibition in *ibi1-1* (Fig. 2). Surprisingly, *(R)*-β-homoserine, (*R*)-β-aminopentanoic acid, *(R/S)*-β-aminohexanoic acid, *(R/S)*-β-aminoheptanoic acid and β-glutamic acid all induced statistically significant levels of *Hpa* resistance, even though they did not repress *ibi1-1* growth (Fig. 2), suggesting alternative perception mechanisms. Of these five β-amino acids, *(R)*-β-homoserine (RBH) showed the strongest resistance-inducing activity, reaching near complete levels of protection at 0.5 mM and 1.5 mM in the soil (Fig. 2). Therefore, subsequent experiments focused on characterization of RBH-induced resistance (RBH-IR).

Role of *IBI1* in RBH-IR

In order to examine the role of IBI1 in RBH-IR, we compared WT (Col-0), *ibi1-1* and *IBI1*-overexpressing *35S:IBI1-YFP* plants (T3; homozygous) for RBH-IR against *Hpa* (Fig. 3a). Although BABA failed to induce resistance in *ibi1-1*, RBH induced WT levels of resistance in *ibi1-1* (Fig. 3a). Thus, despite the fact that our interaction models suggested potential binding of RBH to the (L)-Asp-binding pocket of IBI1 (Fig. S2), RBH-IR does not require IBI1, which is supported by our finding that RBH does not repress *ibi1-1* growth (Fig. 2). Nonetheless, the *IBI1*-overexpressing plants showed near complete levels of resistance following RBH treatment, which was similar to the level of protection induced by BABA (Fig. 3a). Hence, elevated *IBI1* expression acts additively or synergistically on the defense mechanisms underpinning RBH-IR against *Hpa*.

RBH primes defense efficiency of callose

IBI1-dependent resistance is associated with augmented efficiency of callose to halt *Hpa* colonization (Zimmerli *et al.*, 2000; Ton *et al.*, 2005; Luna *et al.*, 2014). To test whether RBH-IR is associated with a similar defense mechanism, 2-wk-old seedlings were soil-drenched with increasing concentrations of RBH, and evaluated for the efficiency by which callose halts *Hpa* colonization at 2 dpi. Epi-fluorescence microscopy of calcofluor/aniline blue-stained leaves revealed that RBH augments callose efficiency in a dose-dependent manner, reaching similar levels at 1.5 mM RBH to plants pre-treated with 0.5 or 1.5 mM BABA (Fig. 3b). Because RBH-treated plants did not deposit enhanced callose in the absence of *Hpa* (Fig. S3), we conclude that RBH, like BABA, primes deposition of resistance-enhancing callose.

RBH-IR operates independently of SA signaling

Because SA-dependent resistance is effective against *Hpa* (Thomma *et al.*, 1998; Ton *et al.*, 2002), we quantified the effect of RBH on expression of the SA-inducible marker gene *PR1*. Leaves of water- and RBH-treated plants (Col-0) were sprayed with water (control) or 0.5 mM SA, and analyzed for SA-induced *PR1* gene expression at different time-points after treatment.

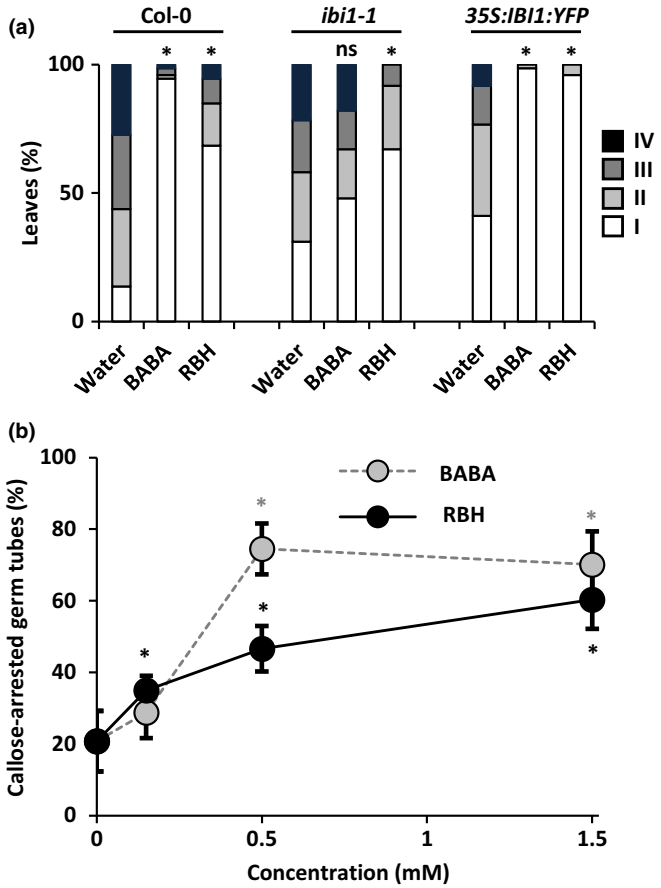

Fig. 3 (*R*)-β-homoserine (RBH)-induced resistance in *Arabidopsis thaliana* against *Hyaloperonospora arabidopsidis* (*Hpa*) acts independently of IMPAIRED IN BABA-INDUCED IMMUNITY 1 (IBI1) and is associated with increased defense efficiency of callose. (a) β-Aminobutyric acid (BABA)- and RBH-induced resistance against *Hpa* in Col-0, *ibi1-1* and *ibi1-1 35S:IBI1:YFP* plants (YFP, yellow fluorescent protein). Two-week-old plants were soil-drenched with water, 0.5 mM BABA, or 0.5 mM RBH, and challenge-inoculated with *Hpa* 2 d later. Shown are percentages of leaves ($n = 70$–80) in different *Hpa* colonization classes at 5 d post-inoculation (dpi). For details, see legends to Figs 1 and 2. Statistically significant differences in class distribution compared to water-treated controls are indicated (Fisher's exact test): *, $P < 0.05$; ns, not statistically significant. (b) Efficiency of callose deposition to arrest *Hpa* colonization. Three week-old Col-0 plants were soil-drenched with increasing concentrations of RBH or BABA, and challenged 2 d later with *Hpa*. Shown are mean percentages of callose-arrested *Hpa* germ tubes at 2 dpi (± SEM; $n = 9$). Statistically significant differences in RBH- (black) and BABA- (gray) treated plants compared to water-treated controls (0 mM) are indicated (Student's *t*-test): *, $P < 0.05$.

RBH did not induce *PR1* expression directly, nor did it augment or accelerate SA-induced *PR1* expression (Fig. S4a). Furthermore, RBH-IR against *Hpa* was unaffected in both the SA induction mutant *sid2-1* (Nawrath & Metraux, 1999), and the SA response mutant *npr1-1* (Cao *et al.*, 1994; Fig. S4b). To account for the possibility that disease protection by RBH is caused by direct toxicity to *Hpa*, plants were treated with RBH at 2 dpi with *Hpa*, as described previously for BABA (Zimmerli *et al.*, 2000). Like BABA, this post-inoculation treatment had no statistically significant effect on *Hpa* colonization (Fig. S5). Thus,

RBH-induced protection against *Hpa* is based on plant-mediated resistance that operates independently of SA signaling.

RBH-IR against *Hpa* partially relies on priming of camalexin induction

Arabidopsis disease resistance relies on pathogen-induced production of metabolites with defense signaling and/or antimicrobial activity (Ahuja *et al.*, 2012). To search for additional mechanisms underpinning RBH-IR against *Hpa*, we profiled a range of defense-related metabolites in water- and RBH-treated plants after mock and *Hpa* inoculation, using UPLC-TQD. In 2-wk-old plants, RBH did not directly induce SA or the phytoalexin camalexin, which are effective in mounting resistance against *Hpa*. Furthermore, both SA and camalexin showed statistically significant levels of induction at 3 dpi with *Hpa* (Table S1). However, although SA induction was similar between water- and RBH-treated plants, camalexin induction was strongly augmented in RBH-treated plants (Fig. 4a; Table S1). To determine whether this priming of camalexin production contributes to RBH-IR, we compared RBH-IR levels between WT (Col-0) plants and the camalexin-deficient *pad3-1* mutant (Zhou *et al.*, 1999). Although the *pad3-1* mutation did not abolish RBH-IR, the mutant showed a statistically significant reduction in efficiency of RBH-IR across a range of RBH concentrations (Fig. 4b). Thus, RBH-IR against *Hpa* relies partially on priming of *PAD3*-dependent camalexin.

RBH-IR against *Hpa* acts through different mechanisms than (L)-α-homoserine-induced resistance in the *dmr1-1* mutant

Arabidopsis mutants in the chloroplastic homoserine kinase (HSK) gene *DMR1* accumulate (L)-α-homoserine (LAH), which induces SA-independent resistance against *Hpa* (van Damme *et al.*, 2009). This *dmr1*-induced resistance is based on endogenous accumulation of LAH, is associated with increased callose deposition, and can be reverted by genetic overexpression of *DMR1*, encoding chloroplastic HSK (van Damme *et al.*, 2009). Although α - and β-amino acids have fundamentally different chemistries and, therefore, rarely have similar biological activities, we investigated whether RBH-IR acts through a similar mechanism as LAH-induced resistance (LAH-IR) in the *dmr1-1* mutant. First, we compared resistance-inducing activities of RBH and LAH after exogenous soil drench application at increasing concentrations. As shown in Fig. S6a, RBH-IR showed partial effectiveness at 0.15 mM and complete resistance at 0.5 mM, whereas LAH-IR was ineffective at 0.15 and 0.5 mM, and only showed a relatively weak induced resistance response at the highest concentration of 1.5 mM. Hence, RBH is an order of magnitude more potent in eliciting resistance to *Hpa* than LAH. Second, we examined whether RBH enhances endogenous LAH accumulation by affecting homoserine kinase activity of DMR1. To this end, we tested the effects of genetic overexpression of the *DMR1* gene on RBH-IR. Although *DMR1* overexpression abolished LAH-induced resistance in L*er eds1-2 dmr1-1* plants, it did not reduce RBH-IR (Fig. S6b). Hence, RBH does not trigger endogenous accumulation of resistance-inducing concentrations of LAH by blocking homoserine kinase activity. However, it is still possible that RBH and LAH share the same immune receptor(s) and elicit a similar immune response. To address this possibility, we tested the contribution of camalexin to LAH-IR. In contrast to RBH-IR, which is partially compromised by the

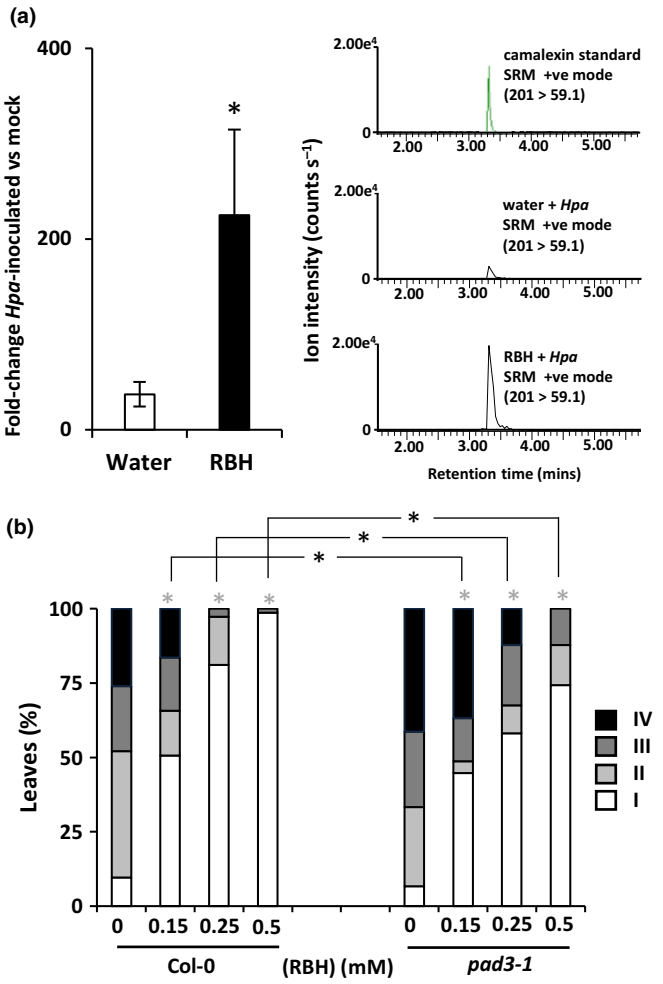

Fig. 4 (*R*)-β-homoserine (RBH)-induced resistance in *Arabidopsis thaliana* against *Hyaloperonospora arabidopsidis* (*Hpa*) depends partially on priming of camalexin induction. (a) Relative quantification of camalexin in extracts from RBH- and water-pretreated plants during *Hpa* infection. Two-week-old plants were soil-drenched with water or RBH (0.5 mM) and challenged 2 d later with *Hpa*. Leaf samples were collected at 3 d post-inoculation (dpi), and analyzed by ultra-performance liquid chromatography coupled to triple quadrupole mass spectrometry (UPLC-TQD) in selected reaction monitoring (SRM) mode. Shown are mean fold-changes in camalexin concentrations between mock- and *Hpa*-inoculated plants for primed (RBH) and unprimed (water) plants (± SEM; $n = 8$). Statistically significant differences to the water-treated control are indicated (Student's *t*-test): *, $P < 0.05$. Right panels show representative UPLC-TQD traces. (b) RBH-induced resistance (RBH-IR) against *Hpa* in Col-0 and the camalexin-deficient *pad3-1* mutant, across a range of RBH concentrations. For details, see legends to Figs 1 and 2. Black asterisks, statistically significant differences between Col-0 and *pad3-1* treated in a similar way; gray asterisks, statistically significant differences compared to the water control within each genotype (Fisher's exact test; $P < 0.05$).

pad3-1 mutation (Fig. 4b), LAH-IR was not reduced by the *pad3-1* mutation (Fig. S6c). Hence, camalexin plays no role in LAH-IR, which supports previous results by van Damme *et al.* (2009), reinforcing our conclusion that RBH and LAH induce, at least partially, different immune responses.

RBH-IR against *Hpa* is not caused by perturbations in branched-chain amino acids

Apart from *dmr1-1*, other mutations causing accumulation of L-Asp-derived branched-chain amino acids have been reported to boost *Hpa* resistance (Stuttmann *et al.*, 2011). To examine whether enhanced accumulation of these amino acids plays a role in RBH-IR, we profiled quantities of RBH (positive control), L-Asp, L-Lysine, L-(iso)leucine, L-threonine, L-methionine and L-α-homoserine at 24 and 48 h after soil-drench treatment with RBH. Leaves of RBH-treated plants contained high RBH concentrations (Fig. S6d), illustrating that RBH is rapidly taken up by the roots and transported to the leaves. However, apart from a small reduction in L-methionine at 24 h after RBH treatment, none of the other branched-chain L-amino acids, including LAH, showed a statistically significant change upon RBH treatment. We therefore conclude that RBH-IR does not act via the changes in endogenous accumulation of L-Asp-derived branched-chain amino acids.

RBH primes JA- and ET-dependent defense against the necrotrophic fungus *Plectosphaerella cucumerina*

In order to investigate whether RBH induces resistance against pathogens other than *Hpa*, we quantified RBH-IR against *Plectosphaerella cucumerina* (*Pc*), which adopts a necrotrophic lifestyle when inoculated at high spore densities (Petriacq *et al.*, 2016a). Compared to water-treated control plants, RBH-treated plants (0.5 mM) showed a statistically significant reduction in lesion diameter and percentage of spreading lesions at 7 dpi (Fig. 5a). Quantification of single copy DNA of *Pc* relative to host plant DNA at 7 dpi confirmed that this disease suppression is based on reduced fungal colonization (Fig. S7). To account for direct biocidal activity of RBH to *Pc*, fully colonized agar plugs were placed on PDA medium with 0 or 0.5 mM RBH and examined for hyphal outgrowth. RBH did not reduce fungal colony size after 4 d of growth (Fig. S8a), excluding biocidal activity as a causal factor for RBH-induced protection against *Pc*. Because JA controls resistance against necrotrophic pathogens (Thomma *et al.*, 1998; Ton *et al.*, 2002), we quantified JA-dependent expression of *VSP2* and *PDF1.2* in water- and RBH-treated plants at different time-points after challenging leaves with mock or 0.1 mM JA solution (Fig. 5b). Although *VSP2* and *PDF1.2*

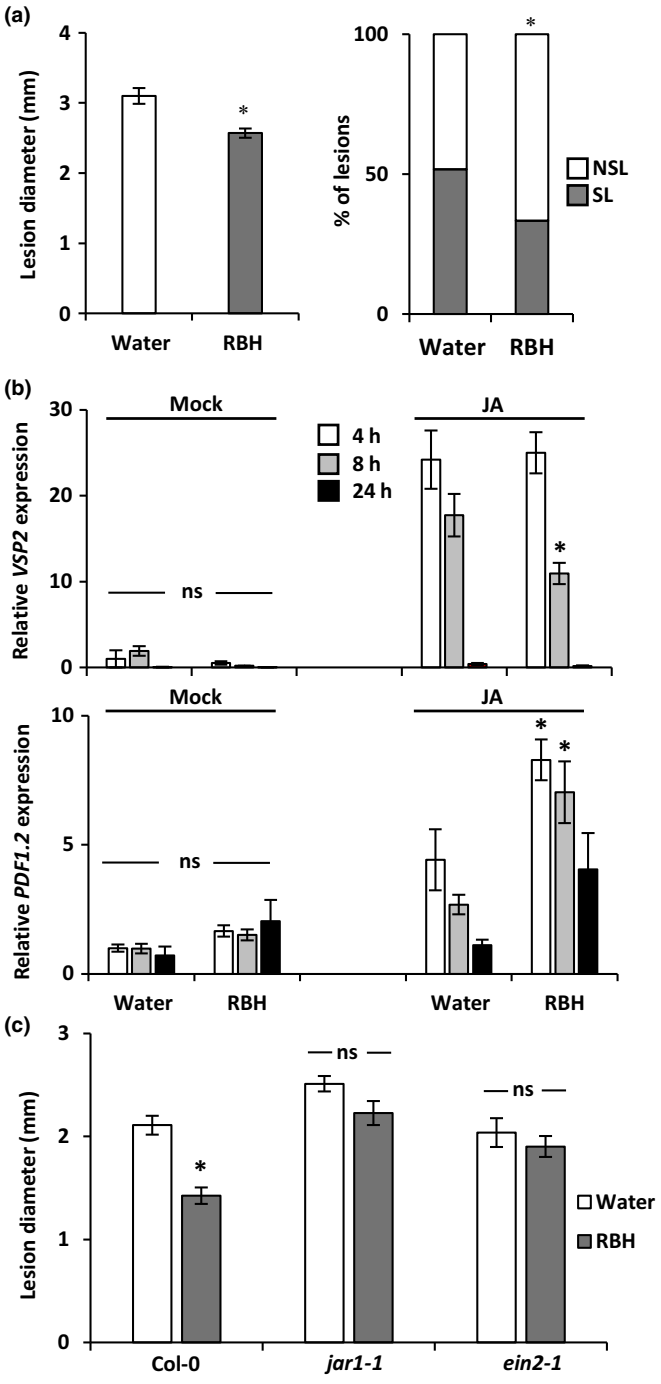

Fig. 5 (*R*)-β-homoserine (RBH) primes jasmonic acid (JA) and ethylene (ET)-dependent defenses in *Arabidopsis thaliana* against necrotrophic *Plectosphaerella cucumerina* (*Pc*). Five-week-old plants were soil-drenched with water or RBH (0.5 mM) and challenged 2 d later with *Pc* or JA. (a) Quantification of RBH-induced resistance (RBH-IR) in wild-type plants (Col-0) against *Pc*. Shown are mean lesion diameters (± SEM; $n = 60$; left), and percentages of spreading lesions (SL) and nonspreading lesions (NSL; right) at 7 d post-inoculation (dpi). Statistically significant differences compared to water-treated controls (Student's *t*-test (left) or Pearson's χ^2 test (right) are indicated: *, $P < 0.05$. (b) Quantification of *VSP2* (upper panel) and *PDF1.2* (lower panel) gene expression in water- and RBH-treated plants (Col-0) at 4, 8 and 24 h after challenge treatment of the leaves with water (mock) or JA (0.1 mM). Shown are average transcript levels (± SEM; $n = 3$), relative to control-treated mock-inoculated plants at 4 h after challenge. Statistically significant differences between water- and RBH-treated plants are indicated (Student's *t*-test): *, $P < 0.05$; ns, not statistically significant. (c) RBH-IR in Col-0, the JA-insensitive *jar1-1* mutant, and the ET-insensitive *ein2-1* mutant. Statistically significant differences to water-treated controls are indicated (Student's *t*-test): *, $P < 0.05$; ns, not statistically significant.

are both inducible by JA, the *PDF1.2* gene is co-regulated by ET (Penninckx *et al.*, 1998), whereas the *VSP2* gene is repressed by ET (Lorenzo *et al.*, 2004). RBH did not induce expression of both genes directly, but repressed *VSP2* induction at 8 h after JA treatment, whereas it augmented *PDF1.2* induction at 4 and 8 h after JA treatment (Fig. 5b). This gene expression pattern suggests that RBH primes the ET-dependent branch of the JA response pathway, which controls resistance against necrotrophic fungi (Lorenzo *et al.*, 2004; Pré *et al.*, 2008; Ahmad *et al.*, 2011). To gain further evidence for this mode of action, we quantified RBH-IR in the JA-insensitive *jar1-1* mutant and ET-insensitive *ein2-1* mutant (Fig. 5c). In contrast to the WT (Col-0), both mutants failed to develop statistically significant levels of RBH-IR against *Pc* at 7 dpi, reinforcing the notion that RBH-IR against this fungus is based on priming of JA- and ET-dependent defenses (Fig. 5c). Interestingly, however, although RBH primed the induction of *PAD3*-dependent camalexin by biotrophic *Hpa* (Fig. 4; Table S1), it did not augment camalexin induction after challenge with *Pc* (Fig. S9). This indicates that RBH primes distinct defense mechanisms against necrotrophic and biotrophic pathogens.

RBH does not majorly affect plant growth and global plant metabolism

Although RBH did not visibly affect agar-grown Arabidopsis (Fig. 2), we investigated potential nontarget effects of RBH at later developmental stages by comparing RGR in 3- to 4-wk-old plants after soil-drenching with increasing concentrations of RBH or BABA. As expected, BABA reduced RGR in a dose-dependent manner, which was statistically significant at concentrations of 0.15 mM and higher. Conversely, RBH did not reduce RGR at any of the concentrations tested (Fig. 6a). To assess nontarget effects of RBH on global plant metabolism, we performed untargeted metabolic profiling of leaf tissues by UPLC-Q-TOF at 3 d after soil-drench treatment. BABA-treated plants showed 38 differentially abundant ions in comparison to water-treated plants, whereas RBH-treated plants showed only four differentially abundant ions (Welch's *t*-test, $P < 0.01$; Fig. 6b). Although putative identities of differentially abundant ions at one time-point before pathogen challenge provide limited information about the underpinning resistance mechanisms (Table S2), the more than nine-fold disparity in differentially abundant ions illustrates that RBH has a relatively minor impact

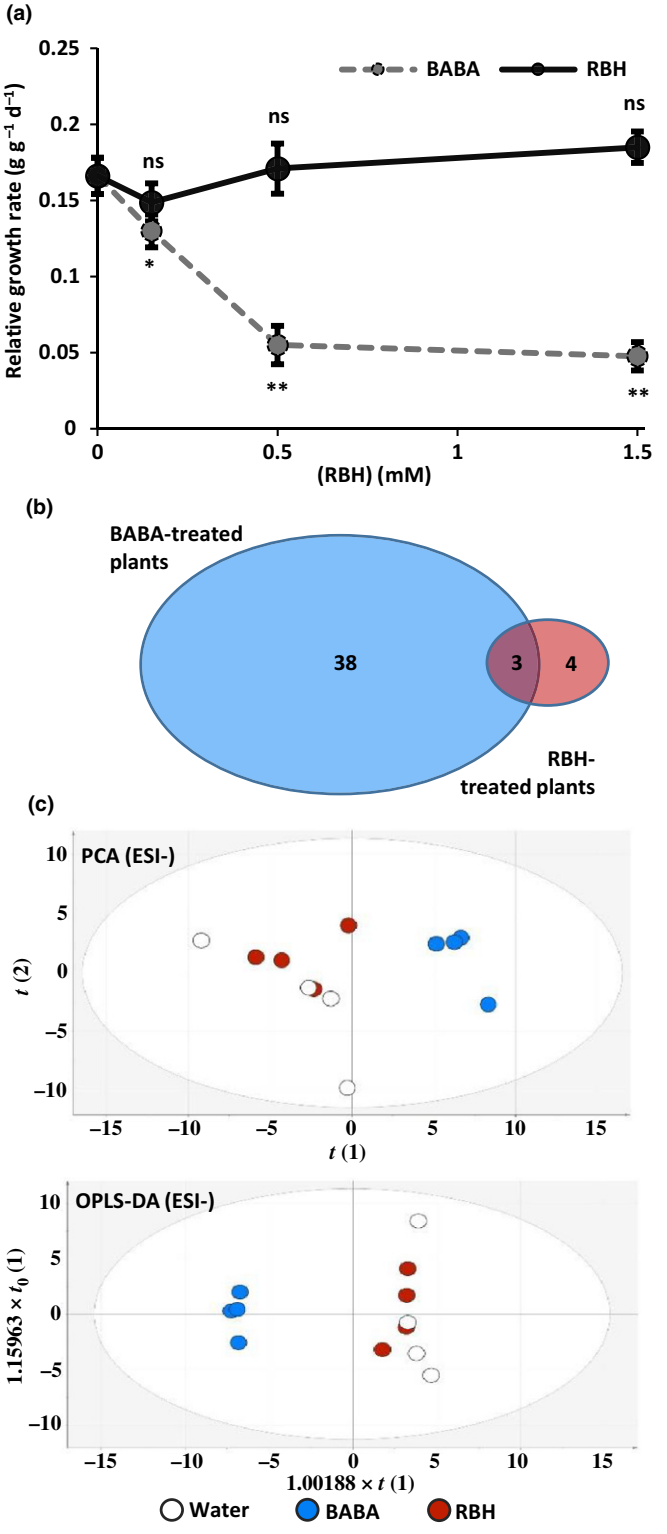

Fig. 6 Nontarget effects of (*R*)-β-homoserine (RBH) and β-aminobutyric acid (BABA) on growth and metabolism of *Arabidopsis thaliana*. (a) Relative growth rates (RGR) of 23- to 30-d-old plants (Col-0) after soil-drench treatment with increasing concentrations of BABA and RBH. Shown are mean values (± SEM; $n = 20$). Statistically significant differences between treatments and water-treated controls (0 mM) are indicated (Student's *t*-test): *, $0.01 < P < 0.05$; **, $P < 0.01$; ns, not statistically significant. (b) Untargeted ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry (UPLC-Q-TOF) analysis of differentially abundant metabolic markers in RBH- (red) and BABA- (blue) treated plants. Leaf material was collected from 26-d-old plants at 3 d after soil-drench treatment with water (control), 0.5 mM BABA, or 0.5 mM RBH. Venn diagram shows numbers of differentially abundant ions (*m/z* values) in RBH- and/or BABA-treated plants (Welch's *t*-test; $P < 0.01$). Data were obtained in negative electrospray ionization mode (ESI-). (c) Multivariate statistical analysis metabolic profiles from UPLC-Q-TOF analysis. Upper panels show unsupervised principal component analysis (PCA) of *m/z* intensities from UPLC-Q-TOF ($R^2X = 0.44$, $Q^2 = 0.11$). Lower panels show orthogonal partial least squares-discriminant analysis (OPLS-DA) ($R^2X = 0.408$, $R^2Y = 0.498$, $Q^2 = 0.286$).

on global plant metabolism in comparison to BABA (Fig. 6b). Subsequent multivariate statistical analysis of data supported this notion: unsupervised principal component analysis (PCA) revealed separate clustering of samples from BABA-treated plants relative to samples from water- and RBH-treated plants (ESI-: $R^2X = 0.44$, $Q^2 = 0.11$; Fig. 6c). More stringent supervised orthogonal partial least-square discriminant analysis (OPLS-DA) revealed a similar clustering pattern (ESI-: $R^2X = 0.408$, $R^2Y = 0.498$, $Q^2 = 0.286$), and failed to separate samples from RBH- and water-treated plants (Fig. 6c). Because OPLS-DA of untargeted Q-TOF data is suitable for detection of subtle shifts in plant stress metabolism (Petriacq *et al.*, 2016a,b), we conclude that the nontarget effects of RBH are negligible in comparison to those of BABA.

RBH induces resistance in tomato without nontarget effects on growth

In order to investigate whether RBH is effective in an economically important crop species, we quantified RBH-IR in *S. lycopersicum* against *Botrytis cinerea* (*Bc*), a necrotrophic fungus that affects global fruit and vegetable production. Sixteen-day-old seedlings (cv Micro-Tom) were soil-drenched with 0.5 mM RBH and inoculated with *Bc*. At 4 dpi, RBH-treated plants showed a 35% reduction in mean necrotic lesion diameters compared to water-treated plants, whereas the percentage of spreading lesions by *Bc* was reduced from 83% in water-treated plants to 34% in RBH-treated plants (Fig. 7a). RBH did not reduce *in vitro* growth of *Bc* (Fig. S8b), confirming that the observed disease suppression is plant-mediated. To examine whether RBH reduces vegetative growth of tomato, we compared RGRs between water-, BABA- and RBH-treated plants over a 4-d period after soil-drenching treatment. Consistent with previous results (Luna *et al.*, 2015), 0.5 mM BABA reduced relative growth rate (RGR) by 51% compared to water-treated plants, which was statistically significant (Fig. 7b). By contrast, 0.5 mM RBH did not reduce RGR, even though it was sufficient to induce resistance against *Bc* (Fig. 7a). Hence, RBH induces resistance in tomato against an economically damaging disease without affecting vegetative growth.

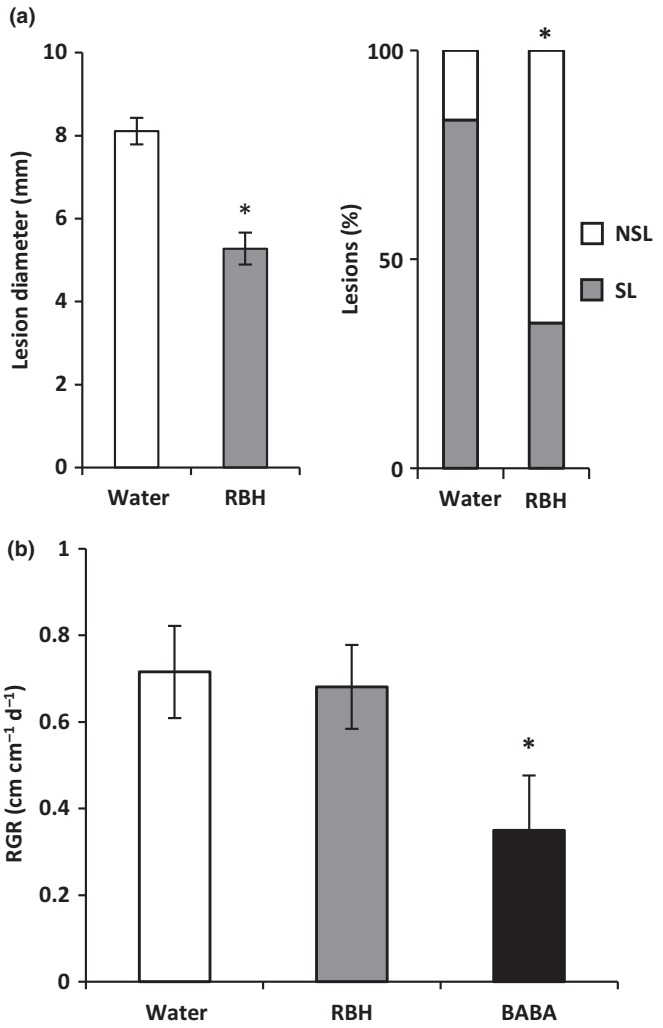

Fig. 7 (*R*)-β-homoserine (RBH) induces resistance in tomato against *Botrytis cinerea* (*Bc*) without affecting growth. Sixteen-day-old tomato (cv MicroTom) seedlings were soil-drenched with water or β-amino acids (0.5 mM), and examined for induced resistance against *Bc* and relative growth rate (RGR). (a) RBH-induced resistance (RBH-IR) against *Bc*. Shown are mean lesion diameters per leaf (± SEM; $n = 16$; left) and percentages of spreading lesions (SL) vs nonspreading lesions (NSL; right) at 4 d post-inoculation (dpi) ($n = 96$). Statistically significant differences compared to water-treated controls are indicated (left, Student's *t*-test; right, Pearson's χ^2 test): *, $P < 0.05$). (b) Mean RGR values (± SEM; $n = 8$) over a 4-d interval after chemical treatments. Statistically significant differences between treatments and water-treated controls are indicated (Student's *t*-test): *, $P < 0.05$.

Discussion

Nontarget effects on plant growth have hampered agricultural exploitation of chemical plant defense activators (van Hulten *et al.*, 2006; Walters & Heil, 2007). In the case of β-aminobutyric acid (BABA), plant growth repression is caused by inhibitory binding to aspartyl-tRNA synthetase (AspRS) enzymes (Luna *et al.*, 2014). Furthermore, BABA is not metabolized quickly in plant tissues (Jakab *et al.*, 2001; Slaughter *et al.*, 2012), increasing the likelihood that the compound accumulates as a chemical residue in crop products. Because BABA inhibits AspRS enzymes (Luna *et al.*, 2014), which are ubiquitous in both prokaryotic and eukaryotic organisms, it follows that this chemical is unsuitable for crop protection purposes. Although numerous other studies have reported new resistance-inducing chemicals over recent years (Bektas & Eulgem, 2015), few provide additional information about their mode of action, their effectiveness in different plant species, their effectiveness against pathogens with different infection strategies, and their nontarget effects on plant growth and metabolism. This additional knowledge is critical for integration of new priming agents in crop protection schemes.

In the present study, we identified (*R*)-β-homoserine (RBH) as a novel plant protection agent that primes distinctly regulated immune responses against pathogens with different infection strategies (Figs 2–5), is effective in different plant species

(Fig. 7a), and has minimal nontarget effects on plant growth and metabolism (Figs 2, 6, 7b). The polar nature of RBH makes it particularly suitable for protection of hydroponically cultivated glasshouse crops via the nutrient supply stream. Indeed, we have shown that RBH is effective in tomato against economically damaging grey mold disease (*Botryitis cinerea*, *Bc*; Fig. 7a). This basic knowledge paves the way for subsequent translational research about the efficiency of RBH under commercial growing conditions and its compatibility with other plant protection and production strategies.

Using site-directed mutagenesis of the *IMPAIRED IN BABA-INDUCED IMMUNITY 1* (*IBI1*) gene, we demonstrated that perception of BABA requires the L-Asp-binding domain of IBI1 (Fig. 1). Accordingly, we performed *in silico* docking studies to select putative BABA analogues with comparable binding affinities to this domain, of which the majority docked in a similar orientation as (*R*)-BABA (Fig. S2). Experimental validation of these models revealed two compounds, *(S)*-β-homoserine and β-alanine, which mimicked BABA activity for induced resistance against *Hyaloperonospora arabidopsidis* (*Hpa*) and selective growth inhibition of *ibi1-1* (Fig. 2). Although this validates our modeling approach, the level of induced resistance by these analogs remained relatively weak, thereby offering limited value for application as crop protection agents. It should be noted, however, that the docking models did not always predict the biological activities of the compounds. For instance, the models suggested that L-threo-3-methylaspartic acid, (*R*)-β-aminopentanoic acid, (*R*)-β-aminohexanoic acid and (*R*)-β-aminoheptanoic would bind with high affinity to the L-Asp-binding domain of IBI1 and with similar orientation as R-BABA. However, the experimental assays revealed that these compounds induced relatively weak resistance to *Hpa* and they failed to repress *ibi1-1* growth (Fig. 2). This discrepancy could be explained by rapid metabolic breakdown of compounds by the plant, resulting in weak AspRS inhibition that is insufficient to induce growth inhibition of *ibi1-1* seedlings. However, the models also predicted high affinity binding of (*R*)-β-glutamic acid and RBH to IBI1 with different molecular orientations than R-BABA. The experimental assays, however, revealed that these compounds induced resistance to different degrees without concurrent growth repression of *ibi1-1*.

Although it is possible that the resistance-inducing activity of IBI1 is not critically dependent on binding orientation of amino acid ligands, our subsequent experiments provide multiple lines of evidence that the resistance response to RBH operates independently of IBI1. First, RBH-induced resistance (RBH-IR) to *Hpa* was unaffected by the *ibi1-1* mutation (Fig. 3a), indicating that the compound is perceived by a different receptor than IBI1. Second, although BABA is known to prime salicylic acid (SA)-dependent gene expression in Arabidopsis (Zimmerli *et al.*, 2000; van Hulten *et al.*, 2006), RBH did not induce nor prime SA-inducible *PR1* gene expression (Fig. S4a). Third, unlike BABA (Ton & Mauch-Mani, 2004), RBH primed jasmonate/ethylene (JA/ET)-dependent defense against *Plectosphaerella cucumerina* (*Pc*) (Fig. 5b). Fourthly, pretreatment with RBH did not affect camalexin induction after *Pc* inoculation (Fig. S9), and augmented camalexin accumulation against *Hpa* (Fig. 4a), whereas BABA pretreatment reduces pathogen-induced camalexin accumulation (Ton & Mauch-Mani, 2004). Finally, BABA and RBH had profoundly different impacts on global plant metabolism. Although unsupervised PCA of ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry (UPLC-Q-TOF) data was sufficient to visualize dramatic shifts in metabolism after BABA treatment, more stringent supervised orthogonal partial least-square discriminant analysis (OPLS-DA) failed to distinguish metabolic patterns between RBH- and control-treated plants (Fig. 6c). This indicates that RBH, unlike BABA, has no major impact on plant metabolism, which is reinforced by the results from our targeted hydrophilic interaction liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry (HILIC-Q-TOF) analysis of L-Asp-derived amino acids (Fig. S6d). Hence, RBH primes plant defense via different pathways than BABA, and does not incur undesirable nontarget effects on plant growth and metabolism.

Although nonproteinogenic β-amino acids have long been regarded as rarities in biological systems, an increasing body of evidence suggests that they play important roles as natural stress signals and/or antibiotics (Kudo *et al.*, 2014). Apart from the recent discovery that BABA accumulates as a low-abundance plant stress signal (Thevenet *et al.*, 2016), β-alanine has been reported to serve as a precursor of the osmoprotectant β-alanine betaine in Plumbaginaceae (Rathinasabapathi *et al.*, 2001). Furthermore, the tyrosine aminomutase TAM1 was recently found to mediate JA-dependent production of β-tyrosine in rice, which has antimicrobial and allelopathic activities (Yan *et al.*, 2015). We did not detect antimicrobial activity of RBH against *Hpa*, *Pc* or *Bc* (Figs S5, S8), nor could we detect RBH in untreated Arabidopsis (Fig. S6b). However, we cannot exclude the possibility that RBH is produced in plants under specific stress conditions. RBH is taken up rapidly from roots to leaves (Fig. S6d), where it primes broad-spectrum defense mechanisms, including callose, camalexin accumulation and JA/ET-dependent defenses (Figs 3–5). Strikingly, nonpathogenic rhizobacteria have been reported to elicit similar systemic priming of callose and JA/ET-dependent defenses as RBH (Van der Ent *et al.*, 2009). In this regard, it is tempting to speculate that RBH is produced in the rhizosphere as an induced systemic resistance-eliciting compound. Future research into the chemistry of rhizosphere communities may therefore cast light on the ecological relevance of RBH.

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Author contributions

J.T. conceived the project; W.B., R.E.S., E.L., B.C., V.F., P.P. and J.T. planned and designed the research; W.B., R.E.S., E.L., M.S., V.F., J.T. and P.P. performed experiments and analyzed data; and W.B. and J.T. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Impact of IBI1 mutagenesis on plant growth.

Fig. S2 Computational docking models of IBI1–ligand interactions.

Fig. S3 Direct effects of BABA and RBH on callose deposition.

Fig. S4 Role of SA signaling in RBH-IR against *Hpa*.

Fig. S5 Post-inoculation effect of RBH on *Hpa* growth.

Fig. S6 Role of branched-chain amino acid metabolism in RBH-IR.

Fig. S7 qPCR quantification of *P. cucumerina* biomass.

Fig. S8 Effect of RBH on *in vitro* pathogen growth.

Fig. S9 Effect of RBH on *P. cucumerina*-induced camalexin.

Table S1 Quantification of defence signaling compounds during RBH-IR against *Hpa*

Table S2 Significantly accumulated putative metabolites after RBH/BABA treatment

Methods S1 Arabidopsis accessions.

Methods S2 Chemical treatments.

Methods S3 Site-directed mutagenesis.

Methods S4 AspRS binding simulations.

Methods S5 Induced resistance assays.

Methods S6 Relative growth rate assays.

Methods S7 Untargeted reversed phase UPLC-MS analysis.

Methods S8 Targeted HILIC UPLC-MS amino acid profiling.

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